NARCOSIS STUDIES AND OXYGEN POISONING OF MICE

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ABSTRACT

During the period since our last report, the Laboratory of Aerospace Biology has been preparing for the critical experiments in our search for a mechanism by which narcotic gases alter metabolism. The main thrust of this effort has been made by David Clarkson, a pre-doctoral candidate, who is exploring possible sites of action by narcotic and anesthetic gases in isolated electron transport particles. Using the relative activities of the NADH-oxygen, NADH-ferricyanide, succinate-cytochrome C and succinate-NAD oxidoreductase systems as parameters, David has been testing the relative potency of volatile anesthetics. His article in this report describes those results and outlines the methods to be used in carrying on this work with the inert gases: helium, nitrogen, argon, nitrous oxide, krypton, and xenon.

Included in this report are the manuscripts of two papers resulting from work by Dr. Christopher Schatte. Testing the relative ability of human subjects to contract and repay an oxygen debt while in the narcotic versus alert state, he found that narcosis induced by 33% nitrous oxide increased the size of the oxygen debt contracted and the amount of oxygen required to repay it during recovery. Narcosis did not appear to alter the ability of cells to utilize oxygen but apparently did affect the rate of change in metabolic rate when going from the resting to the active state.

The results of collaborative work with the Department of Physiology and Biophysics is contained in the manuscript entitled "Relative Susceptibility of Altitude-Acclimatized Mice to Oxygen Poisoning". Mice acclimatized to sea level (760 mm Hg), 5000 feet (632 mm Hg) or 15,000

feet 437 mm Hg) for from one to eight weeks were found to be more susceptible to convulsion and death as a function of altitude acclimatization when tested in hyperoxic environments. There were no reasonable explanations for the connection between hypoxia and oxygen poisoning but several practical implications for persons living at altitude are discussed.

A BIOCHEMICAL STUDY OF INERT GAS NARCOSIS II

Introduction:

It is currently believed that most clinical anesthetics [e.g. diethyl ether, halothane (inhalation) and barbituric acid derivatives (ionic)] are primarily membrane active compounds (1). The mechanism of action of these two classes of anesthetics, however, may differ. Inhalation anesthetics because of their hydrophobic nature and neutral charge tend more to affect a disordering of the hydrophobic interior of the membrane and consequently may affect the activity of various membrane bound enzymes and ion and water permeability (2,3,4). On the other hand ionic anesthetics due to their charge (under physiological conditions) tend to be more surface active and very likely neutralize membrane surface charges thereby affecting the binding of ions to the membrane and as a consequence the permeability of these ions would also be affected (3,5,6).

The so-called inert gases (including nitrogen) have long been known to effect a narcotized state in vivo, but usually at partial pressures far exceeding those of the conventional inhalation anesthetics (Table I). This, most likely, is attributable to their comparatively low lipid solubility. These data are also presented in Table I. That inert gases and inhalation anesthetics have a common mechanism is by inference, based primarily on their solubilities and little direct evidence exists.

To elucidate the mechanism of anesthetic action many investigators have chosen some form of membrane system, e.g. plasma membrane, microsomal (endoplasmic reticulum) and mitochondria. Mitochondria are a particularly good model in that they are a comparatively well defined system obtainable in large quantities with reproducible characteristics. Electron transport particles, which can be prepared from mitochondria, are more preferable

Table I. (1,7)

	Anesthetic Pressure	Solubility*
Argon	20	0.144
Helium	-----	0.015
Krypton	2.9	0.44
Neon	-----	0.0196
Nitrogen	29	0.0632
Nitrous Oxide	0.9	1.56
Xenon	0.85	1.9
Halothane	0.0087	370.
Methoxyflurane	0.0023	932.

* Bunsen coefficients for gas/olive oil system at 25°C

since they contain only a few but highly integrated, readily assayable enzyme systems.

Recent studies on mitochondria and electron transport particles have shown that inhalation anesthetics are particularly effective in inhibiting the electron flow through complex I (NADH-CoQ oxidoreductase) (8,9). In intact mitochondria these anesthetics concomitantly reduce the P/O ratio and energy coupling (respiratory control). Whether this reduction is exclusively a result of complex I inhibition has not been adequately demonstrated. At anesthetic concentrations up to a slight excess of what are deemed to be clinical levels, these effects are essentially reversible indicating at least inhibition does not involve covalent bonding. Because they are quite soluble in lipid, their effects are believed to be entirely ascribable to a disordering of an otherwise highly organized system. This is supported by the observations of rather marked increases in mobility of fatty acyl chains within the hydrophobic regions of synthetic and plasma membranes (10,11) in the presence of various anesthetics. Further, complex I is known to contain at least 4 iron-sulfur centers in addition to the dehydrogenase and flavine mononucleotide (12). These centers which are in a highly ordered configuration are necessary for electron transport through the complex and are isolated from the aqueous environment in a medium of low dielectric constant, namely lipid. (This is also true to a certain degree of the remainder of the respiratory chain). The lipid not only serves as an insulator but probably is structural as well.

Consequently, one can visualize at least two processes which would significantly alter the electron flux through complex I: 1) a solubilization

of the lipid fraction which may increase the mobility of the electron carriers such that they are no longer in a configuration conducive to electron transfer, or 2) the solubilization process may be sufficient to allow water from the aqueous environment to enter the complex raising the dielectric constant and thereby effectively insulating various elements of the chain from one another. The latter condition is not very likely since it would not be reversible, at least in vitro, and thus would contradict available data. The former mechanism seems non compatible with current data. In this case the irreversible effect of high anesthetic concentration can be explained by lipid extraction which is irreversible beyond a certain point (1,8,9).

If inert gases belong to the class of inhalation anesthetics, their affect on the behavior of complex I should be very similar. As a consequence of their low lipid solubility this type of behavior could be anticipated to occur only at high pressure which for argon and nitrogen is in excess of 10 atmospheres and for krypton and xenon at pressures less than 10 atmospheres. Helium and neon have no demonstrable anesthetic properties even at extremely high pressures (several hundred atmospheres). This may be due to their anomalous behavior with regard to compressibility, activity (13,14) and low lipid solubility.

Data are presented which verify the reported effects of two popular inhalation anesthetics, halothane and methoxyflurane, on respiratory chain activity.

Methods and Materials:

As indicated the experiments of necessity will be conducted at pressures greater than one atmosphere. For this purpose, a special cuvette has been constructed which can be pressurized in excess of 100 ATA. The cell

(designed specifically to fit the Beckman DK-2A) has been machined from type 316 stainless steel bar stock. The ends are threaded to accept the window retaining rings. The windows are 10 x 5 mm quartz discs and are sealed to the cell with teflon gaskets. With a total cell volume of about 1 ml the "active" area of these windows is about 0.28 cm^2 and the light path between the inside faces is 1 cm ± 2%. The inside top of the cell has been domed by milling to capture any gas bubbles which would otherwise be trapped in the light path. A depression has also been milled into the bottom of the cell to accept a small magnetic stirring bar to provide rapid mixing of the cell contents.

Continuous with the domed region are injection and gas ports. The injection port is equipped with a standard silicon septum used in gas chromatography. The septum is supported below by a stainless steel back-up plate to minimize the pressure on the septum and above by a coned needle guide which is a portion of the retaining nut. The injection port allows the addition of material to initiate a reaction once the cell is pressurized. A standard 1000 series Hamilton Gaslight syringe equipped with a plunger guide and stops is used, since they are reasonably accurate, relatively inexpensive and will withstand the operating pressure (15).

Externally mounted on either side of the cell is a jacket through which liquid can be pumped to maintain a constant cell temperature. Additionally, a small well has been drilled into the top of the cell which will accept either a thermocouple or other temperature transducer for monitoring the cell temperature. These features are particularly important since the DK-2A is not equipped with a constant temperature compartment and it is anticipated that cell heating may be significant as a consequence of

gas compression.

The gas coolant connections and cell are mounted on a heavy gauge (0.1"] aluminum alloy plate which indexes the cell precisely in the cell compartment and permits a ready exchange of this cell with other cell holders for more conventional use. Additionally, the DK-2A has been modified so that an external recorder can be used. This is a necessity, since the instrument was designed for scanning and not for monitoring activity at a single wavelength.

The cell is pressurized by a small hand-operated hydraulic pump, the outlet of which is attached to a 1 liter reservoir. In operation the reservoir is half filled with the hydraulic fluid (ethylene glycol) and the remainder with the gas to be tested. The head space of the reservoir is connected to the bulkhead fitting on the cell supporting plate by a short piece of hydraulic hose and a double-sealing quick disconnect fitting. Between this fitting and the cell are a tee for evacuating and filling the reservoir with the desired gas, a valve to isolate the cell from the hydraulic system, and another tee for a pressure transducer to monitor the cell pressure. All of the plumbing on the cell plate is 1/8" stainless steel tubing mated to the various components with stainless steel Swaglock fittings.

In practice all components of a particular assay are transferred to the cell except the substrate required to initiate the reaction. The cell is sealed and brought to the desired pressure, time allowed for temperature equilibration and/or preincubation and the reaction is initiated as described above. Helium is used as a reference gas for all assays. Data will primarily be evaluated in terms of their lipid solubilities and

concentration corrected for activity (14). Gases to be tested include nitrogen, argon, nitrous oxide, krypton, and because of the small volume of the system xenon may also be tested without undue expense.

The model system chosen to study the effects of inert gases on complex I activity is phosphorylating electron transport particles (ETP_p) prepared from rat liver mitochondria by the method of Smith and Hansen (16). This method has been modified by shortening disruption time to two minutes at 60% output (about 84 watts into the half-inch tip) of a Branson model W140C Sonifier. ETP_p prepared in this manner and stored frozen (-10°C) are useable for one to two weeks. The following enzymatic activities are utilized to gauge the effect of inert gases on respiratory chain activity: NADH-oxygen, NADH-ferricyanide, succinate-cytochrome C, and succinate-NAD oxidoreductases. The use of ATP-Pi exchange is being contemplated.

The NADH oxidase activity is measured by the classical method of monitoring the change in absorbance at 340 nm at 25°C. Malonate (1 mM) is included in the assay medium for the reason discussed below. The specific activity is calculated from the linear portion of the curve.

NADH dehydrogenase (NADH-ferricyanide oxidoreductase) activity is assayed by a modification of the method of Singer (17). Unlike the assay for the soluble preparation of this enzyme, rotenone (100 ng) is added to the assay preparation to block electron flow through the remainder of the respiratory chain. [Antimycin (3 µg) was also tested and results were not significantly different]. This is important, since it was observed that there are at least two sites on the respiratory chain where electrons can be transferred to ferricyanide. One has a K_m of about 120 µM and the other a K_m of about 4 mM with respect to ferricyanide and 150 µM NADH. The site

with the higher K_m is thought to be cytochrome C, and that with the lower value is the dehydrogenase. However, in the presence of rotenone a K_m of 1 mM is consistantly observed, a value which agrees with that determined by King, et. al. (18). In addition to rotenone the assay medium also contains 0.3 M mannitol, 0.004 M $MgCl_2$, 0.04 triethanolamine and 0.001 M malonate and is adjusted to pH 7.8. The malonate is included to suppress succinate dehydrogenase activity; this enzyme will also reduce ferricyanide at about the same rate as NADH dehydrogenase. Succinate is used as one of the necessary stabilizing agents in the ETP_p storage medium; without it the particles deteriorate very rapidly. Each assay requires 5-10 µg of ETP_p protein (depending primarily on their age) and is initiated by addition of the appropriate amount of ferricyanide. The reduction of ferricyanide is followed at 420 nm at 25°C and the rate extrapolated from a 12 second segment of the absorbance curve (usually about 10 seconds after initiation).

It should be emphasized that this assay does not measure the full potential of the dehydrogenase, since the reduction of ferricyanide is the rate limiting step. It is, however, the only reliable assay for this enzyme.

Succinate-cytochrome C oxidoreductase activity is measured after a modified method of Tisdale (19). The assay medium is the same as used for NADH dehydrogenase minus the malonate and rotenone. Sixty µg of ETP_p protein are preincubated for 2.5 minutes in the assay medium with 5 mM succinate at 25°C. The assay medium is then made 1.5 mM in KCN and the reaction initiated with 500 µg of ferricytochrome C (horse heart). The reduction of cytochrome C is followed at 550 nm and the specific activity with respect to succinate oxidized is determined from the linear portion

of the curve.

Although ATP-Pi exchange assay has not been used, a convenient method is that described by Pullman (21). Up to about 300 µg of ETP_p protein is incubated in an assay medium containing 0.006 M $MgCl_2$, 20 mM Pi pH 7.8, and 0.05 µCi ^{32}P (carrier free). The reaction is initiated with the addition of 10 mM of ATP and incubated at 25°C for about 15 minutes. The reaction is terminated with an equal volume of 6 N $HClO_4$ and extracted 3 times x 10 seconds with ethyl acetate and a final extraction with diethyl ether following the addition of 1/2 volume of 0.2 M sodium molybdate. An aliquot of the remaining aqueous phase is taken for counting. Exchange activity is calculated as the ratio of the total activity of ATP presumed to be in aqueous phase to the specific activity of the Pi in the original incubation medium. Mannitol has been omitted in this assay, since it is not compatible with $HClO_4$.

The rationale for these assays are the following. The NADH-ferricyanide oxidoreductase assay approximately measures the full potential of the dehydrogenase and serves as a check on the quality of the preparation. Its activity can be correlated with the activity of the corresponding oxidase and reverse electron transport activities. The NADH oxidase serves as the workhorse in these experiments, because it is principally by the assay of its activity that the influence of inert gases will be quantified. The reverse flow serves as a check on foreward flow to verify that the observed effect resides in complex I and is not a reflection of activity of the remainder of the chain. Succinate-cytochrome C oxidoreductase serves a similar role by monitoring the activity of the chain above complex I to the level of cytochrome C. The terminal oxidase activity can be inferred only

indirectly, since the amounts of ETP_p protein available are not sufficient for a polarographic assay. The ATP-Pi exchange assay serves to test the hypothesis that the inert gases and several of the clinical inhalation anesthetics are general inhibitors of complex I like amytal as opposed to the more specific action of rotenone and Piercidin A. Ernster, et al. (22) has reported that amytal markedly reduces the activity of phosphorylation site I as measured by ATP-Pi exchange. On the other hand rotenone did not elicit a noticeable effect at concentrations producing a complete inhibition of electron flow. This suggests that amytal not only inhibits electron transport, but may be an uncoupling agent as well.

The following procedures were used to establish base-line data for the supression of respiratory chain activity by inhalation anesthetics and ultimately the inert gas studies. Anesthetic was introduced into the assay medium by one of two methods. In the first method, the stock solution was prepared by adding an amount of anesthetic to a volume of assay medium to a final concentration of 3 mM. The amount was calculated from the molecular weights and densities of the anesthetics. Following the addition of anesthetic the vials containing the medium were sealed with foil-lined caps and vigorously agitated for 30 seconds with a Vortex mixer. These solutions were used immediately and kept sealed except when aliquots were withdrawn.

Alternately saturated solutions were prepared by mixing 2 ml of the anesthetic with 20 ml of the assay medium. These preparations were allowed to stand for 24 hours, with periodic shaking, for equilibration. Aliquots of 0.5 ml were then extracted with 2 ml of redistilled tetrachloroethane for quantification by gas-liquid chromatography. Samples were run on

10% EGA on chromsorb-WHP 80/100 mesh in a 1/8 " x 6 foot stainless steel column at 90°C mounted in a Hewlett-Packard model 7620A gas chromatograph equipped with flame-ionization detectors. Nitrogen was used as a carrier gas.

In either case desired concentrations of the anesthetics were prepared from the stock by dilution with anesthetic-free assay medium in a spectrophotometer cuvette. The required volume of anesthetic solution was added after the ETP_p, the cuvette was immediately sealed and inverted several times to mix the contents. Within 30 seconds the substrate or acceptor was added to initiate the reaction and the cuvette resealed.

Results and Discussion:

Data are presented in Table II which were obtained in this laboratory and serve to verify some of the observed effects reported in the literature for two of the more popular inhalation anesthetics. Data for succinate-cytochrome C, NADH-oxygen, and NADH-ferricyanide oxidoreductases are the result of 2 or more replications. The data for succinate-NAD oxidoreductase were not replicated. Assays were conducted as described above.

Generally these values agree with those reported in the literature, however, some exceptions are to be noted. It was surprising to note the effect of these anesthetics on succinate-cytochrome C oxidoreductase. However, some caution is in order regarding the current data. The concentrations of anesthetics are higher than what is generally accepted as a clinical level. The activiation of this portion of the chain can be explained by the fact that ETP are turned inside out. In normal configuration cytochrome C is located near the outer surface of the inner

Table II. Effect of inhalation anesthetics on enzymatic activity on the respiratory chain

Enzyme	Anesthetic		% Inhibition
NADH-O_2 oxidoreductase	none		0
	halothane	0.5 mM	45
		1.5 mM	45
		2.5 mM	54
	methoxyflurane	0.5 mM	42
		1.5 mM	50
		2.5 mM	59
NADH-ferricyanide oxidoreductase	none		0
	halothane	2.3 mM	0
	methoxyflurane	2.3 mM	0
Succinate-cytochrome C oxidoreductase	none		0
	halothane	2.5 mM	7
	methoxyflurane	2.5 mM	-10
Succinate-NAD oxidoreductase	none		0
	halothane	0.5 mM	78
		1.5 mM	93
		2.5 mM	100
	methoxyflurane	0.5 mM	43
		1.5 mM	90
		2.5 mM	100

membrane while this relationship is reversed in ETP (23). Therefore, it would seem that the anesthetic may have effectively solubilized the ETP membrane either making the electron transfer site more accessible to exogenous cytochrome C or perhaps exposing a new electron donar site. This also follows from the very high lipid solubility of methoxyflurane which is higher than that of halothane.

Another interesting finding is that the degree of inhibition of the NADH-oxygen and succinate-NAD oxidoreductases while showing a dependency on anesthetic concentration shows only a very limited dependency on lipid solubility. This is contrary to the findings of Cohen and McIntyre (8) who reported a very close correlation of the degree of inhibition of intact mitochondria respiration, anesthetic concentration and lipid solubility. Our studies were performed with ETP and may possibly explain the difference and would suggest that structure or configuration of the inner membrane is an important factor. This alludes to the presummed sidedness effect observed with the succinate-cytochrome C portion of the respiratory chain discussed above. In the only other study known to have been performed with ETP (9) titration curves were presented for halothane only and data for other anesthetics tested are not sufficiently definitive to permit a meaningful evaluation of the effect of lipid solubility.

Until data are collected which are to the contrary it is believed that the seemingly nonsignificant effect of lipid solubility is artifactual. This belief is based on the following observation. During the course of performing these assays, it was noted the effect of a given concentration of anesthetic was not always serially reproducible. This lack of conformity was ultimately traced to the rather high volatility of these anesthetics. For example, it was observed that the inhibitory

effects of these anesthetics could essentially be reversed by leaving the cuvette open to room air for a few minutes; this occurs more quickly with methoxyflurane than with halothane. Presumably the major losses occurred during the transfer of the anesthetic-containing medium to the cuvette. Although various attempts were made to limit this problem, none of the handling procedures were ever very satisfactory. The most effective way to resolve this difficulty is to use gas-phase equilibration. In this approach both the stock assay medium and assay medium in the cuvette would be maintained under a gas mixture composed of air combined with the desired amounts of anesthetic. The anesthetic concentration can be monitored by either gas chromatography or infrared spectrophotometry. This is currently being tried.

The next few weeks will bring to a close these inert gas studies. A more detailed and concluding write-up is to appear in the next status report.

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A P P E N D I X A

This Appendix contains the manuscript entitled "Effects of N_2O Narcosis on the Contraction and Repayment of an Oxygen Debt" which has been submitted to the editors of Aerospace Medicine for publication.

EFFECTS OF N_2O NARCOSIS ON THE CONTRACTION AND REPAYMENT OF AN OXYGEN DEBT

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EFFECTS OF N_2O NARCOSIS ON THE CONTRACTION AND REPAYMENT OF AN OXYGEN DEBT

Abstract

The oxygen deficit (VO_2 Df), oxygen debt (VO_2 Dt) and difference [VO_2(Dt-Df)] were measured in five male and three female subjects during and after exercise while breathing either air or a normoxic mixture of 33% N_2O and nitrogen. With the exception of a higher ($P \leq .05$) respiratory quotient at rest in N_2O, there were no statistically significant differences for oxygen consumption, carbon dioxide production, expired gas volume, heart rate or blood lactate while breathing N_2O during rest, exercise or recovery. An appreciably but not statistically greater VO_2 Df was found in N_2O along with significantly ($P \leq .05$) greater VO_2 Dt and VO_2 (Dt-Df). It was speculated that N_2O narcosis did not affect the ability to utilize oxygen but that the response to the greater oxygen need of exercise may have been slowed with a concomitantly greater depletion of stored high energy compounds.

oxygen deficit; oxygen debt; nitrous oxide narcosis; oxygen consumption; blood lactate; exercise

Introduction

Metabolic changes associated with a state of inert gas narcosis can, in most cases, be attributed to the semisomnolent condition usually induced by the narcotic gas. Although histotoxic hypoxia is probably not a cause of inert gas narcosis, there are certain reports which suggest that narcosis may cause an altered availability of oxygen to the cell or its ability to use it.

Schatte et al (7) observed that rats breathing a mildly hypoxic (17.2% oxygen) mixture of argon at one atmosphere had a significantly reduced oxygen consumption and a degree of hypoxia apparently more severe than animals breathing similar mixtures of helium or nitrogen. Bradley and Dickson (1) reported a consistent, and occasionally significant, decrease in oxygen consumption of human subjects breathing 30% N_2O both at rest and during exercise. Conversely, Webber (12) found an increased oxygen uptake under similar conditions. His work is consistent with the findings of Theye and Michenfelder (11) who reported an increase in canine cerebral oxygen uptake while breathing 70% N_2O.

If oxygen flux in the narcotic state is different from than when alert, it is conceivable that the oxygen kinetics during aerobic exercise might be altered in divers breathing a narcotic gas.

The present study was undertaken to evaluate the contraction and repayment of oxygen debt during mild narcosis such as that which might be experienced by divers breathing compressed air at seven to ten ATA.

Methods

The experimental design was based on that used by Mohler and Armstrong (6) whose paper should be consulted for a detailed theoretical explanation of the protocol. Briefly, the approach involved the measurement of VO_2 in the resting state, during a short period of exercise sufficient to induce an oxygen debt, and during an arbitrary period of recovery. The dashed line in Figure 1 depicts a representative VO_2 curve for such a sequence. Considering the relatively steady state $\dot{V}O_2$ measured during the final two minutes of exercise as equal to that necessary to meet the energy requirements of the exercise without drawing upon other energy sources, the total oxygen requirement can be calculated and is depicted by the solid line. The difference between this theoretical requirement and the measured oxygen consumption is the oxygen deficit (VO_2 Df). Similarly, the total oxygen consumed during recovery in excess of that required at rest is the oxygen debt (VO_2 Dt).

Five male and three female subjects aged 21-29 years and weighing 53.6 - 90.9 kg were tested once each while breathing air or a mixture of nitrous oxide, nitrogen and oxygen at ambient pressure (632 mm Hg). Chromatographic analysis of the mixtures indicated that N_2O concentrations ranged between 32.7 and 34.1%, oxygen concentrations between 20.8 and 21.5% with the balance nitrogen. The subjects were non-smokers in relatively good physical condition but not in active atheltic training. Only one subject had breathed a narcotic gas previously. There was no formal training except for a single preliminary run to familiarize the subjects with the protocol.

The subjects made their two experimental runs on Tuesday and Thursday of the same week. The sequence of exposure to air or N_2O was reversed in half the subjects to reduce pattern effects.

The subjects were allowed only a light meal two hours prior to the experiment. All wore light clothing, blinders, and earphones supplying music during the experiments. The test gas was breathed in through 3.75 cm flexible tubing into a modified Otis-McKerrow valve (Warren E. Collins Co.) and out through a 10 liter gas meter (American Standard Co.). Mixed expired gas was sampled continuously from the exhaust port of the gas meter and the oxygen (Servomex paramagnetic analyzer) and carbon dioxide (Beckman LB-1 infrared analyzer) concentrations recorded on strip charts.

The experiments began at 1000 hours with the subjects in the supine position breathing the test gas (room air or the N_2O mixture from a Douglas bag). The resting phase lasted for 30 minutes to allow good equilibration of the test gas with the subject's body. Rest was followed by five minutes exercise on a bicycle ergometer (Godart) at 60 rpm. Workloads of 175 W for males and 100 W for females were found to increase $\dot{V}O_2$ from five to eight times above resting level without causing the subjects undue discomfort. Recovery was arbitrarily measured for 10 minutes because a return to resting $\dot{V}O_2$ was found to nearly occur for most subjects during this time.

During each minute of the last five minutes of rest, five minutes of exercise and 10 minutes of recovery, the expired gas volume, EKG and expired gas concentrations were measured. Immediately prior to and after

exercise and at 5, 10 and 15 minutes recovery, a blood sample was drawn from the antecubital vein in an arm which remained immobile throughout the experiment. All samples were immediately deproteinized in iced perchloric acid and stored frozen until analysis for blood lactate (Sigma bulletin 826-UV).

Results

The mean values for VO_2, VCO_2, RQ, heart rate, VE and blood lactate are listed in Table 1. With the exception of resting RQ, there were no significant differences ($P \leq .05$) between air and N_2O during rest, exercise or recovery. The lack of statistical significance was due to large variation among the subjects probably as a result of differences in sex, physical condition, and the response to narcosis.

The oxygen deficit (VO_2 Df) and debt (VO_2 Dt) were calculated using the following formulae:

$$VO_2 \text{ Df} = 5[\dot{V}O_2 \text{ (steady state)}] - \Sigma\ VO_2 \text{ (exercise)}$$

$$VO_2 \text{ Dt} = \Sigma\ VO_2 \text{ (recovery)} - 10[\dot{V}O_2 \text{ (rest)}]$$

In table 2, it can be seen that the mean VO_2 Df, VO_2 Dt and the difference between them, VO_2 (Dt-Df), was appreciably greater in N_2O than in air, with the differences between VO_2 Dt and VO_2 (Dt-Df) being statistically reliable ($P \leq .05$).

The slightly higher mean steady state $\dot{V}O_2$ in N_2O contributed to the greater mean deficit incurred while narcotic. Since the mean total oxygen consumption during exercise was essentially the same in both gases, it follows that there was a lower VO_2 in N_2O during the first three minutes of exercise. Thus, it would seem that the differences in VO_2 Df between the two gases may have been partially a function of the rate of change from rest to exercise. Narcosis did not depress the ability to attain a steady state $\dot{V}O_2$ at least equal to that in air.

The greater oxygen debt in N_2O was, in part, due to the fact that $\dot{V}O_2$ was higher at the end of exercise and had to fall to a lower resting level during recovery in N_2O than in air. The greater mean oxygen consumption during recovery in N_2O may have resulted in part due to this fact.

Discussion

The data do not provide evidence for a mechanism by which narcosis might alter VO_2 Df, VO_2 Dt, VO_2 (Dt-Df) but certain considerations are pertinent to possible explanations.

The incurrence of an oxygen deficit during the transition from a resting to an exercise steady state has been attributed to a lag in cardiovascular response to exercise (3), the time required for metabolic readjustment of muscle cells (5) and the time for the muscle cells to develop a need for more oxygen (8). In humans anesthetized with halothane, the addition of 75% N_2O to the breathing mixture slightly increased circulating norepinephrine levels, increased peripheral and pulmonary vascular resistance and slightly reduced cardiac output (10). In unanesthetized subjects, most of these effects were absent. Since our subjects were not anesthetized and were much less narcotic than that experienced when breathing 75% N_2O, we are inclined to believe that circulatory deficiency was minimal in N_2O. Perhaps the slight but consistently reduced mean heart rate observed was a manifestation of the degree of cardiovascular impairment during narcosis.

With regard to the effects of N_2O on metabolic reactions, the evidence is conflicting (2,4,11,13) and, for the most part, probably not applicable to the present results. It may be pertinent, however, that the preponderance of evidence suggests that analgesic levels of N_2O do not significantly inhibit certain metabolic functions (2) and may actually increase oxygen uptake (11,12). The facts that mean steady state $\dot{V}O_2$ during exercise was actually higher in N_2O and that total oxygen consumption

during exercise was the same in both gases suggest that the ability to use oxygen was not impaired by the narcosis. Rather it appears that the rate of increase in $\dot{V}O_2$ during the first few minutes of exercise was slower in N_2O than in air leading to a greater mean oxygen deficit.

The repayment of oxygen debt includes such processes as lactate oxidation, resaturation of oxyhemoglobin, reduction of oxidized coenzymes, restoration of high energy substances and increasing the oxygen tension in tissue fluids (9). The greater mean debt in N_2O implies that the execution of these processes required more oxygen either as a result of a greater degree of recovery required or a reduced efficiency of repayment in N_2O. Attendant with the slower rate of $\dot{V}O_2$ increase which occurred in N_2O during the first few minutes of exercise, there may have been greater depletion of stored energy compounds during this time since there was less oxygen available to meet the body's energy needs. If this was the case, the greater subsequent repayment of oxygen debt may have been due partially to the larger amount of high energy compounds which had to be replaced.

The probable reasons for VO_2 Dt exceeding VO_2 Df are discussed in detail elsewhere (6) but include such factors as changes in body temperature, metabolic efficiency and the level of resting metabolism as a consequence of the exercise. Whatever the reasons, N_2O increased the size of this difference. There is no immediately apparent reason for this other than to suggest reduced efficiency of debt repayment.

The practical consequences of these findings are unclear but relate to the performance of work while breathing a narcotic gas. The narcosis

induced by 30% N_2O is thought to approximate that experienced when breathing compressed air at seven to ten ATA. If the physiological alterations caused by N_2O in this study are extrapolatable to those attendant with work at these pressures, we suggest that compressed air narcosis may cause a greater VO_2 during recovery from work and perhaps changes in the high energy metabolites in muscle cells. The magnitude of these effects would not appear to be cause for concern although, in combination with other physiologic problems such as the work of breathing and heat balance, may add to the oxygen requirement of a compressed air worker and perhaps predispose him to fatigue.

Table 1. Mean ± S.E. values for eight subjects breathing air or a mixture of N_2O. The resting figures are the average of three consecutive one-minute observations during rest. Those for exercise and recovery are sums of five and ten minutes, respectively.

Parameter	Gas	Rest	Exercise	Recovery
VO_2 ml/kg STPD	Air	4.17 ± .26	122.93 ± 6.64	79.17 ± 5.91
	N_2O	3.83 ± .72	120.78 ± 4.65	89.08 ± 3.27
VCO_2 ml/kg STPD	Air	3.26 ± .20	108.11 ± 6.78	84.70 ± 6.79
	N_2O	3.30 ± .19	119.20 ± 6.40	89.80 ± 4.75
RQ	Air	.80 ± .00	.87 ± .01	1.00 ± .01
	N_2O	.86 ± .00*	.98 ± .01	1.02 ± .01
HR beats	Air	70.67 ± 3.36	684.50 ± 26.09	960.50 ± 44.55
	N_2O	64.66 ± 3.27	662.62 ± 18.08	915.37 ± 48.90
VE liters STPD	Air	8.82 ± .39	261.30 ± 18.79	232.99 ± 21.41
	N_2O	8.75 ± .44	242.40 ± 18.11	229.76 ± 14.44
Blood Lactate mmol/liter	Air	1.55 ± .24	5.77 ± 1.32	7.12 ± 1.19
	N_2O	1.60 ± .24	6.09 ± .73	6.81 ± .77

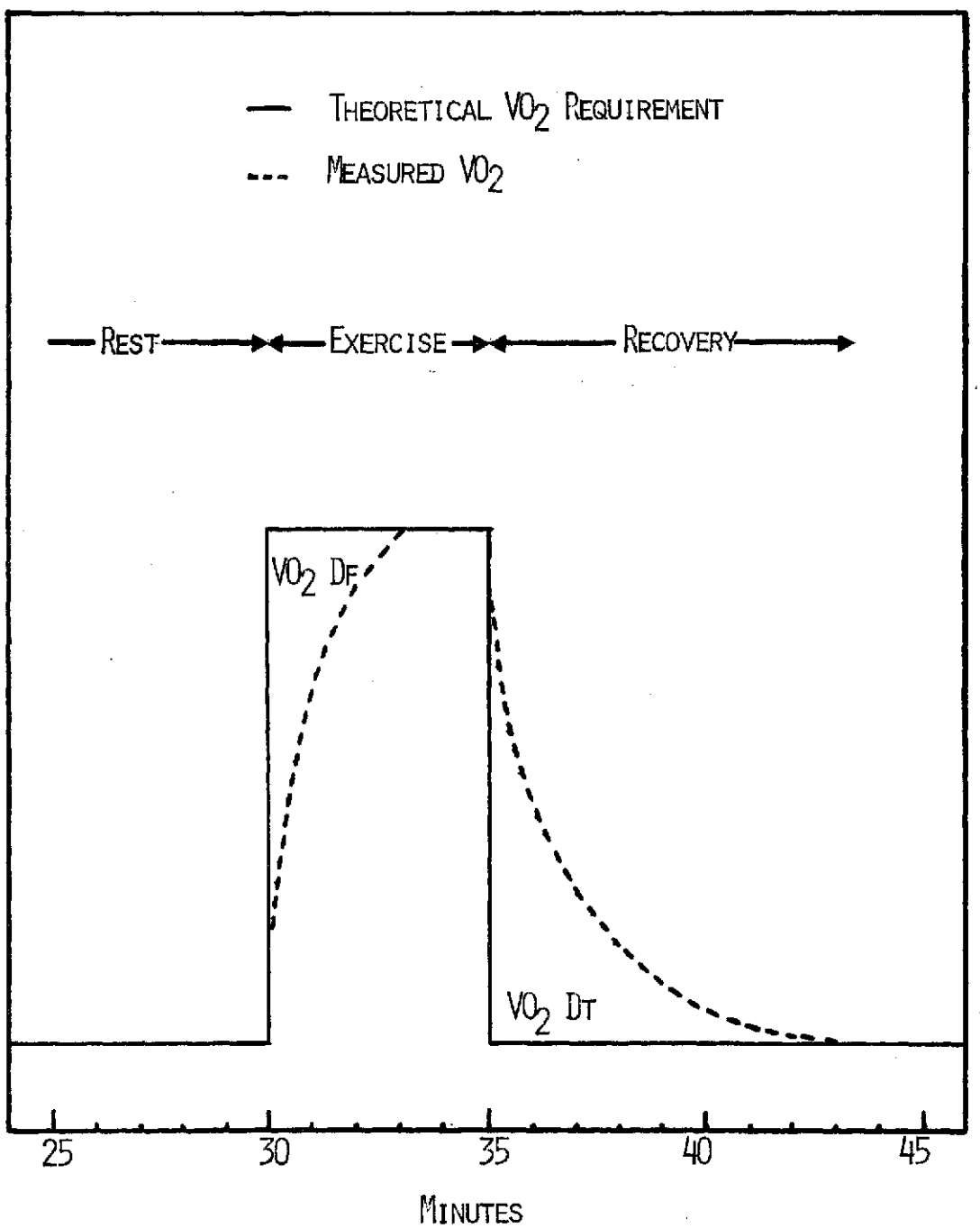
* statistically different from air at $P \leq .05$.

Table 2. Mean ± S.E. values for eight subjects breathing air or a mixture of 30% N_2O. The $\dot{V}O_2$ ss represents the assumed steady state based on the final two minutes of exercise. VO_2 Df and VO_2 Dt are sums. All values are in ml/kg STPD.

Gas	$\dot{V}O_2$ ss	VO_2 Df	VO_2 Dt	VO_2(Df-Dt)
Air	28.20 ± 1.72	20.63 ± 4.45	37.41 ± 4.85	16.78 ± 2.18
N_2O	30.17 ± 1.31	33.66 ± 4.74	50.71 ± 2.37*	34.87 ± 5.28*

* Statistically different from air at $P \leq .05$ using paired "t" test.

Figure 1.


THEORETICAL VO_2 REQUIREMENT
MEASURED VO_2
REST
EXERCISE
RECOVERY
VO_2 DF
VO_2 DT
25
30
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MINUTES

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A P P E N D I X B

This Appendix contains the manuscript entitled "Relative Susceptibility of Altitude-Acclimatized Mice to Acute Oxygen Toxicity" which has been submitted to the editors of the Journal of Applied Physiology for publication. This paper is included because Christopher Schatte is an author and consultant although the work was supported by funds from the Department of Physiology and Biophysics at Colorado State University.

RELATIVE SUSCEPTIBILITY OF ALTITUDE-ACCLIMATIZED MICE TO ACUTE OXYGEN TOXICITY

by

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Hypoxic potentiation of O_2 toxicity

RELATIVE SUSCEPTIBILITY OF ALTITUDE-ACCLIMATIZED MICE TO ACUTE OXYGEN TOXICITY

Abstract

The influence of hypoxic acclimatization at altitudes of 0, 5000, or 15,000 feet on the relative susceptibility to acute oxygen poisoning was determined in 288 adult female mice. After acclimatization periods of one, two, four or eight weeks, the mice were exposed to oxygen at high pressures (OHP) of four, six or nine atmospheres absolute and the times to convulsion and death recorded. A factorial analysis of variance indicated that altitude and OHP level had inverse, linear effects on both parameters. The duration of acclimatization progressively decreased death time and the interval between convulsion and death but did not affect convulsion time. The onset of convulsions and death was independent of body weight. There were significant interactions on the measured parameters between various combinations of altitude, OHP level, and duration of acclimatization. Changes in the metabolism of gamma-aminobutyric acid, high energy compounds and lipids, which are common to both hypoxia and hyperoxia, are discussed but failed to provide a suitable explanation of the results.

altitude; hypoxia; acclimatization; oxygen at high pressures; oxygen poisoning; convulsions; death

Introduction

In addition to the traditional professions of caisson and underwater work, there is increasing exposure of humans to oxygen at high pressure (OHP) during the treatment of certain diseases, radiotherapy and sport diving. A substantial number of these people live at an altitude other than sea-level and are therefore acclimatized to a reduced inspired oxygen tension.

If hypoxic acclimatization prior to a hyperoxic episode alters the individual's susceptibility to the symptoms of oxygen toxicity, it would be necessary to properly adjust exposure times and maximum OHP levels to maintain safe operating conditions. Brauer et al. (1) have reported that rats acclimatized to an altitude of 17,400 feet (382 mm Hg) survived the pulmonary damage attendant with an OHP level of 1.08 atmospheres absolute (ata) more than three times longer than sea-level controls. But at seven ata, the altitude rats convulsed in half the time of controls.

The present study was undertaken to further describe the relationship between hypoxic acclimatization and the relative susceptibility to convulsions and death resulting from exposure to OHP. Specific emphasis was placed on statistically quantifying the influence of and possible interrelationships between a range of acclimatization altitudes, durations of acclimatization and OHP levels.

Methods and Materials

A group of 300 female mice (CFW strain, Carworth Farms) with a mean ± s.d. weight of 19.34 ± 1.71 grams were raised to adulthood at sea level, randomly divided into three groups and placed in three chambers at altitudes of 0 feet (P_B = 760 mm Hg, PO_2 = 159 mm Hg), 5000 feet (P_B = 632, PO_2 = 135) and 15,000 feet (P_B = 437, PO_2 = 89) in air. All were housed under similar conditions of caging, light cycle, and feed (Purina Lab Chow). Once each day, the chambers were brought to ambient pressure (632 mm Hg) briefly for servicing. The ranges of chamber temperatures (26-29°C), relative humidities (19-56%), and CO_2 levels (.06-.19%) were maintained by adjustment of gas flow through the chambers.

At intervals of one, two, four and eight weeks, a randomly selected group of 24 mice was removed from each chamber, weighed, and divided into three groups of eight animals. These groups were exposed to OHP at four, six, or nine ata (60, 90, or 135 psia) in a hyperbaric chamber (volume = 1000 liters) fitted with a 24 compartment wood and wirecloth cage, which allowed an unobstructed view of all subjects. All oxygen exposures had eight mice from each of the three altitude groups, thereby enabling a valid comparison between groups for every OHP test.

Compression rate was 0.66 ata per minute and a flow was maintained through the chamber such that oxygen analyses of the gas entering and leaving the chamber differed by no more than 0.2% (Servomex paramagnetic analyzer).

The time to convulsion for each subject was recorded as the time in minutes between reaching pressure and the onset of full clonic spasm. Time to death was taken as the time in minutes between reaching pressure and the last visible respiratory movement.

Results

The data were analyzed using a factorial analysis of variance for each of the measured parameters of body weight, time to convulsion, and time to death, plus a derived one, the time between convulsion and death. A summary of the statistical results is listed in Table 1. The data for times to convulsion and death were analyzed using the common logarithmic transformations because a plot of these times versus OHP level revealed an exponential relationship. Further, the means and standard deviations of the 36 eight-mouse groups exhibited a constant ratio for all three OHP levels, a condition for which logarithmic transformation of the data is valid and recommended as a more powerful analysis (8).

Body weight increased significantly throughout the experiment but altitude inversely affected the rate of increase, a finding typical of hypoxic exposure (9). An orthogonal breakdown of the sums of squares indicated that both the effects of altitude and duration at altitude were linear functions. In addition to these single factor effects, there were significant interactions between the altitude and duration of acclimatization and between altitude and OHP level. However, these interactions were statistically of lesser magnitude than the single factor effects.

Using correlation and regression tests, body weight was found to have had no effect on any of the other parameters. This agrees with the previously reported findings (1,4) that occurrence of oxygen toxicity is independent of body weight.

The logarithm of time to convulsion varied inversely as a function

of altitude and OHP level and the orthogonal analysis indicated that these relationships were primarily linear (≥90%) with a smaller quadratic component. The duration of acclimatization at altitude had no effect. Statistically significant interactions of lesser magnitude were noted between altitude and OHP level, altitude and duration at altitude, OHP level and duration at altitude, and all three of these factors in combination.

Following a pattern similar to that for time to convulsion, the logarithm of time to death varied inversely as a function of altitude, OHP level and duration at altitude. A significant variation due to duration at altitude resulted from a drop in time to death at eight weeks; there was no significant fluctuation at one, two or four weeks' time. The relationships of time to death to altitude and OHP level were linear with small quadratic components while that for duration at altitude was a combination of linear, quadratic and cubic functions. The linear relationship to time to death is in agreement with previous reports (3) which show an exponential or log-linear pattern for mice over a range of oxygen pressures from 0.2 - 10 ata.

There were statistically significant interactions of lesser magnitude between altitude and OHP level, altitude and duration at altitude, and OHP level and duration at altitude.

Time between convulsion and death represents the interval between the onset of full clonic convulsion and death. There was significant variation of this parameter as a function of OHP level and duration at altitude but the duration effect was somewhat inconsistent. The effect of OHP level was almost entirely linear. A significant interaction also existed between OHP level and duration at altitude.

Discussion

The results indicate that acclimatization to hypobaric hypoxia prior to exposure to oxygen at high pressure enhanced the susceptibility of mice to convulsions and death. Predictably, the OHP level was the primary determinant of times to convulsion and death but altitude also had an inverse, linear effect of lesser magnitude on both parameters. Duration of acclimatization at altitude had no effect on time to convulsion but decreased time to death and time between convulsion and death at eight weeks.

Of particular note is the fact that both altitude and OHP level influenced times to convulsion and death linearly. This allows the influence of either factor to be predicted for a given combination of altitude and OHP exposure.

The results confirm the findings of Brauer et al. (1) that rats acclimatized to 17,400 feet (382 mm Hg, PO_2 = 80 mm Hg) had a lower mean time to convulsion than sea-level controls during exposure to an OHP level of seven ata. The mean decrease in convulsion time for the rats was 50%, which quantitatively agrees with the 40% decrease in our mice maintained at 15,000 feet and exposed to four, six or nine ata.

The mechanism by which altitude acclimatization potentiates the effects of OHP cannot be discerned from our data but three possibilities are pertinent. First, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) has been shown to decrease during exposure to OHP (10) and prior to the onset of drug-induced convulsions (13). Prophylactic treatment with GABA or its metabolic precursors prevents or delays the

onset of convulsions in OHP (11). Hypoxia can increase brain GABA levels but an exposure to less than 8% inspired oxygen or a simulated altitude of 24,000 feet is required to do so in rats (12). If GABA is a key metabolite involved in protection against OHP-related convulsions, it appears that an agent which potentiates the effects of OHP would tend to decrease GABA concentration in the brain. Since hypoxia apparently increases brain GABA and then only under hypoxic conditions of far greater severity than those used in this work, we do not consider a common action of hypoxia and OHP on brain GABA levels to be a reasonable explanation of the present results.

The second possible explanation relates to a common influence of hypoxia and OHP on brain high energy compounds, typified by ATP. Sanders et al. (7) have presented evidence suggesting that OHP- and drug-induced convulsions result from a decrease in brain ATP concentration below a threshold level. Prophylactic treatment with compounds which generate ATP in the tricarboxylic acid cycle, primarily succinate, prevents or delays the onset of convulsions, presumably by maintaining ATP concentration above the threshold value. Acute severe hypoxia (PO_2 = 22-24 mm Hg) did not significantly alter the adenine nucleotide levels in rat brains (5) and we are unaware of any evidence showing that the level of brain high energy compounds is reduced by the degree of hypoxia used here. We therefore cannot reasonably conclude that the hypoxic potentiation of oxygen toxicity in this study resulted from a reduction of brain high energy compounds.

A third consideration concerns lipid metabolism as a factor common

to hypoxia and oxygen toxicity. It can be inferred that the presence of body fat enhances susceptibility to oxygen poisoning (6). Prior starvation affords dramatic protection against it (4). The protective effect of starvation probably results from a decrease in depot lipids since treatment with an anti-lipolytic agent prior to starvation abolishes the protective effect and actually enhances the symptoms of OHP (6). The fact that body weight gain in the present study was reduced as a function of altitude suggests that the altitude-acclimatized mice were in a relative state of starvation when compared with controls. If the weight differences reflected differences in the relative amounts of body lipid, we would expect the altitude mice to have been less, rather than more, susceptible to OHP toxicity. Since this was not the case, it seems unlikely that lipid metabolism, as indicated by relative amounts of body lipid, was a causative factor.

Whatever those mechanisms by which hypoxia influences OHP toxicity may be, there appears to be an OHP level between one and four ata at which hypoxia ceases to protect against oxygen poisoning, as implied in the report by Brauer et al. (1), but rather enhances it. It may be that oxygen pressure at which the predominant, and ultimately fatal, symptom of oxygen poisoning ceases to be pulmonary deterioration and becomes a central nervous system lesion. Thus, it might be speculated that either a single mechanism of hypoxia exists which protects the lungs but is detrimental to the CNS, or that more than one mechanism is operative and the level of OHP determines which one will predominate.

The present results have practical implications in hyperoxic

therapy, underwater diving or any other circumstance in which a person acclimatized to hypoxia becomes exposed to oxygen at raised partial pressure. This is particularly important because animals larger than mice or rats are more susceptible to oxygen poisoning (2) so that they may be affected by altitude to an even greater extent than the mice used in this study. Persons living at altitude who must be treated with hyperbaric oxygen for severe cardiorespiratory disfunction, radiotherapy of tumors, or infectious diseases may be predisposed to the toxic effects of oxygen. Underwater divers living at altitude who breathe compressed air at elevated ambient pressures are subject to a similar risk. This risk may be further potentiated if other factors which predispose to oxygen toxicity (exercise, hypercapnia, high temperature, certain drugs) are also present. It is therefore necessary that further delineation of the interaction between hypoxic acclimatization and OHP exposure be made so that appropriate adjustments in the maximum exposures to a given oxygen pressure can be made on the basis of previous exposure to a particular altitude.

Table 1. Results of a factorial analysis of variance for body weight, time to convulsion, time to death and time between convulsion and death as a function of altitude of acclimatization (1), level of high oxygen pressure (2), duration of acclimatization (3), and all possible combinations of these variables. Values for time to convulsion and time to death are logarithmic transformations. The P value is the minimum level of significance for those Fisher ratios with a random chance probability of 5% or less.

Parameter	Source of Variation	df	ms	p
Body Weight	1	2	106.54	.005
	2	2	.56	
	3	3	437.09	.005
	12	4	11.57	.025
	13	6	14.35	.01
	23	6	.92	
	123	12	5.74	
	error	252	3.66	
Time to Convulsion	1	2	1.317	.005
	2	2	64.994	.005
	3	3	.039	
	12	4	.133	.005
	13	6	.068	.01
	23	6	.056	.025
	123	12	.080	.005
	error	252	.023	
Time to Death	1	2	.124	.005
	2	2	23.575	.005
	3	3	.021	.025
	12	4	.023	.005
	13	6	.020	.005
	23	6	.050	.005
	123	12	.009	
	error	252	.006	
Time between Convulsion and Death	1	2	965.54	
	2	2	84036.50	.005
	3	3	8193.73	.005
	12	4	1523.86	
	13	6	1040.33	
	23	6	8967.82	.005
	123	12	652.88	
	error	252	636.10	

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